Modulation of T and B cell function in Granulomatosis with polyangiitis

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CHAPTER 4

KV1.3 BLOCKADE BY SHK186 MODULATES CD4+ EFFECTOR MEMORY T-CELL ACTIVITY OF PATIENTS WITH GRANULOMATOSIS WITH POLYANGIITIS *IN VITRO*

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Submitted, under revision

ABSTRACT

$CD4^+$ effector memory T cells (T_{EM}) play a key role in the pathogenesis of granulomatosis with polyangiitis (GPA). Interestingly, activation of $CD4^+T_{EM}$ cells is uniquely dependent on the voltage-gated potassium Kv1.3 channel. In this study we aimed to modulate $CD4^+T_{EM}$ cell activity via Kv1.3 blockade using the specific peptide inhibiter, ShK-186. We assessed the effect of ShK-186 on the cytokine production within total $CD4^+$TH cells and $CD4^+$TH cell subsets from GPA patients and age matched healthy controls using flow cytometry. Expression of IFNγ, TNFα, IL-4, IL-17, and IL-21 was significantly increased in $CD4^+$TH cells from GPA-patients compared to HCs. Additionally, ShK-186 normalized the level of cytokine production in $CD4^+$TH cells from GPA-patients in vitro. Furthermore, analysis performed on sorted $CD4^+$T cell subsets including T_{NAIVE}, T_{CM}, T_{EM} and terminal differentiated T (T_{TD}) cells revealed that ShK-186 predominantly inhibited the cytokine production of $CD4^+T_{EM}$ cells leaving other $CD4^+$T cell subsets unaffected. We demonstrate that blockade of Kv1.3 channels by ShK-186 modulates the effector function of $CD4^+$TH cells in GPA-patients in vitro, and predominantly affects cytokine expression by $CD4^+T_{EM}$ cells. Modulation of cellular effector function by ShK-186 may constitute a novel treatment strategy for GPA with high specificity and less harmful side effects.

BACKGROUND

Granulomatosis with polyangiitis (GPA) is the prototype of anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV). GPA is a chronic relapsing systemic autoimmune disease characterized by medium to small vessel vasculitis predominantly affecting the upper and lower respiratory tract and kidneys [1] which may result in life-threatening complications [2]. Current treatment consists of nonspecific immunosuppressive therapy including cyclophosphamide in combination with corticosteroids [3]. More recently, B cell depletion therapy with rituximab has been demonstrated to be equally effective as conventional therapy in inducing disease remission [4, 5]. Unfortunately, for many patients current treatments are unsatisfactory and there is a clear need to identify novel molecular targets to develop more selective and less harmful treatment strategies.

It remains unknown how GPA develops but accumulating evidence indicates a key role for $CD4^+$ T-helper (T_H) cells in disease pathogenesis [6]. In the peripheral blood of GPA patients, a subset of memory $CD4^+$ T_H cells termed effector memory T (T_{EM}) cells were found to be increased during remission [7]. In active disease, $CD4^+$ T_H cells with a memory phenotype have been demonstrated in pulmonary lesions, nasal and renal biopsies of GPA patients [8-12]. Previously, we have reported a marked increase in $CD4^+$ T_{EM} cells in the urinary sediment with a concomitant decrease of circulating $CD4^+$ T_{EM} cells in GPA patients with active renal involvement [13]. These urinary $CD4^+$ T_{EM} cells decreased or disappeared from the urine during remission, which potentially reflects their key role in disease progression and tissue injury [13]. Presumably, migrating $CD4^+$ T_{EM} cells produce inflammatory cytokines (such as interleukin (IL)-17, IL-21, and interferon-gamma (IFNγ)) that may be involved in chronic tissue inflammation and contribute to granuloma formation in GPA patients [14]. Additionally, T cells detected in granuloma of GPA patients have been shown to exhibit increased production of IFNγ and tumor necrosis factor-alpha (TNFα) [10]. Moreover, a skewing towards T_H17 effector cells with an increase in IL-21-producing T_H cells have been demonstrated in peripheral blood of GPA patients [15-17]. Collectively, the data described above indicate that $CD4^+$ T_{EM} cells play a prominent role in the induction and progression of GPA. Therefore, selective targeting of $CD4^+$ T_{EM} cells without impairing other arms of cellular immunity might have value in the treatment of GPA-patients.

Activation of $CD4^+$ T_{EM} cells is uniquely dependent on the voltage-gate potassium Kv1.3 channels [18]. Kv1.3 channels are expressed on T cells in a distinct pattern that depends on the state of activation as well as on the state of differentiation of the given T cell subset [19]. It has been shown that Kv1.3 channels are highly expressed on $CD4^+$ T_{EM} cells (~1500 channels per cell), whereas naïve ($CD4^+$ T_{NAIVE}) and central memory ($CD4^+$ T_{CM}) $CD4^+$ T cells express lower levels of Kv1.3 channels (~250 channels per cell) [20]. Therefore, Kv1.3 channels may serve as an attractive target for specific immunomodulation in T_{EM} cell mediated chronic or autoimmune diseases. Indeed, previous studies have demonstrated that selective blocking of Kv1.3 channels ameliorates disease development in animal models of multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes mellitus (T1DM), and contact dermatitis without compromising protective immune responses to acute infections [21-23].

Accordingly, we hypothesized that selective blocking of Kv1.3 channels on $CD4^+$ T_{EM} cells from GPA patients, using a highly potent peptide inhibitor called ShK-186, reduce their pathogenic

function through modulating their pro-inflammatory cytokine production. To test this hypothesis, we studied the effect of ShK-186 on pro-inflammatory cytokine production of $CD4^+$ T_H cells from GPA patients *in vitro*. Furthermore, we evaluated the effect of ShK-186 on the cytokine production of $CD4^+$ T cell subsets.

MATERIALS AND METHODS

Study population

Twenty-seven GPA patients in remission and 16 age-matched healthy controls (HCs) (5 males and 11 females, mean age of 60 years, range [27 – 77]) were included in this study. The diagnosis of GPA was established according to the definition of the Chapel Hill Consensus Conference [33] and fulfilled the classification of the American College of Rheumatology [34]. Only GPA patients without clinical signs and symptoms of active disease and considered to have complete remission of their disease, as indicated by a Birmingham Vasculitis Activity Score of 0, were included in this study [35]. All patients were PR3-ANCA positive at disease diagnosis. At time of sampling eighteen patients were PR3-ANCA positive as indicate by an ANCA titer ≥1:40. The PR3-ANCA titers were measured by indirect immunofluorescence (IIF) on ethanol-fixed human granulocytes according to the standard procedure as described previously [36]. Twenty-one patients were considered to have generalized disease, and six patients were considered to have localized disease, in which the disease was confined to the upper and lower respiratory tract. None of the patients experienced an infection at the time of sampling as indicated by a median CRP level of 5.8 mg/L. Eight of the twenty-seven GPA patients were treated with maintenance immunosuppressive therapy at the time of blood withdrawal. One GPA patient received azathioprine, five GPA patients received azathioprine in combination with prednisolone, and two GPA patients were treated with low dose prednisolone. Detailed clinical and laboratory characteristics of the patients are summarized in table 1. All patients and healthy controls provided informed consent and the local medical ethics committee of the University Medical Center Groningen approved the study.

Table 1 | Clinical and laboratory characteristics of the GPA-patients at the time of blood sampling

	GPA
Subjects, n (% male)	27 (44%)
Age, mean (range)	61 (34 – 79)
PR3-ANCA positive[1], n (% positive)	18 (67%)
Localized / generalized disease, n (% generalized)	6 / 21 (78%)
CRP (mg/L), median (range)	5.8 (<0.3 – 11)
eGFR ml/min*1,73m^2, median (range)	64 (15 – 91)
Disease duration in years, median (range)	9.5 (1.3 – 30.8)
Number of previous relapses, median (range)	1 (0 – 6)
Non / maintenance immunosuppressive therapy[2], n	19 / 8

[1]ANCA-positive titer ≥1:40, ANCA-negative ≤1:20
[2]immunosuppressive maintenance therapy: azathioprine, azathioprine + prednisolone, or prednisolone.

Sample preparation and *in vitro* peripheral blood stimulation

Lithium-heparinized venous blood was obtained from GPA-patients and HCs. Immediately after blood withdrawal, 2 ml of blood was mixed with 2 ml of RPMI 1640 (Lonza, Basel, Switzerland), supplemented with 50 µg/ml Gentamicin (GIBCO, Life Technologies, Grand Island, NY, USA) and 10 % fetal calf serum. The diluted blood samples were aliquoted into 5 mL polypropylene tubes (Falcon®, Corning incorporated) at 400 uL per tube. Next, the blood samples were pre-incubated in the presence or absence of ShK-186 (dose range [0.1 nM – 100 nM]; Kineta Inc, Seattle, WA, USA) for 1 hour at 37 °C, followed by stimulation with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) and 2 mM calcium ionophore (CaI, Sigma-Aldrich). The cultures were incubated for 16 hours at 37 °C with 5% CO_2. As a negative control, one sample was kept without stimulation. To inhibit cytokine release from cells, 10 µg/ml brefeldin A (BFA; Sigma-Aldrich) was added to each sample.

Immunofluorescence staining of peripheral blood

After stimulation of the peripheral blood, erythrocytes were lysed using ammonium chloride and the cells were washed in wash buffer (PBS containing 1% (w/v) bovine serum albumin (BSA)). T cells were stained with Brilliant Violet 605-conjugated anti-CD3 (Biolegend, San Diego, CA, USA), APC-eF780-conjugated anti-CD8 (eBioscience, San Diego, CA, USA), FITC-conjugated CD45RO (BD Pharmingen™, Franklin Lakes, NJ, USA) and PE-Cy7-conjugated CCR7 (BD Pharmingen™) for 15 minutes at room temperature. Cells were fixed with 100 µl fixation reagent A (Fix/Perm medium A, life technologies, Breda, The Netherlands) for 15 minutes. After washing , cells were resuspended in 100 µl permeabilization reagent B (Fix/Perm medium B, life technologies) and labeled with PerCP-Cy5.5-conjugated anti-IL-4 (Biolegend), APC-conjugated anti-IL-17A (eBioscience), PE-conjugated anti-IL-21 (eBioscience), Alexa Fluor®700-conjugated anti-IFNγ (BD Pharmingen™) and Pacific Blue-conjugated anti-TNFα (Biolegend) for 30 minutes at room temperature in the dark. Finally, the samples were washed and analyzed by nine-color flow cytometric analyses on BD™ LSR II flow cytometer. Data were collected for 5 * 10^5 events for each sample and plotted using Kaluza v1.5a (Beckman Coulter, Brea, CA, USA). Because stimulation reduces the surface expression of CD4 on T cells, $CD4^+$ T cells were identified indirectly by gating CD3-positive and CD8-negating lymphocytes. Gated $CD4^+$ T cells were further displayed as density dot plots for the evaluation of intracellular cytokine production. The unstimulated negative control sample was used to discriminate cytokine producing from non-cytokine producing $CD4^+$ T cell populations.

Purification of $CD4^+$ T_{NAIVE} and $CD4^+$ T_{EM} cells

PBMCs of 5 HCs were used for cell sorting experiments. Cell suspensions were stained for CD3, CD8, CD45RO, and CCR7. $CD4^+$ T cells were gated negatively as CD3-positive and CD8-negative cells and sorted into: $CD4^+$ T_{NAIVE} ($CD45RO^-CCR7^+$), $CD4^+$ T_{CM} ($CD45RO^+CCR7^+$), $CD4^+$ T_{EM} ($CD45RO^+CCR7^-$), and $CD4^+$ T_{TD} ($CD45RO^-CCR7^-$) cell fractions on a MoFLO astrios sorter (Beckman Coulter). The purity of the sorted $CD4^+$ T cell subsets, as determined by a post sort analysis, was > 98% for all sort $CD4^+$ T cell subsets. From each subset, 2.5 * 10^5 cells were incubated in the

presence or absence of ShK-186 (dose range [0.1 nM – 100 nM]; Kineta Inc) for 1 hour at 37 °C, followed by stimulation with 50 ng/ml PMA (Sigma-Aldrich) and 2 mM CaI (Sigma-Aldrich) in the presence of BFA. Following incubation for 16 hours at 37 °C with 5% CO_2, cell were washed, premeabilized, and stained intracellularly for IL-4 (Biolegend), IL-17A, IL-21 (eBioscience), IFNγ (BD Pharmingen™), and TNFα (Biolegend). Finally, the samples were acquired on a BD™ LSR II flow cytometer (BD Biosciences) and data was analyzed using Kaluza 1.5a. Unstimulated samples were used as negative control for setting gates to define cytokine producing cells.

Statistical analysis

Statistical analysis was performed using GraphPad prism v5.0 (GraphPad Software, San Diego, CA, USA). Data are presented as median values or mean ± SEM, as indicated. Data were analyzed with the D'Agostino & Pearson omnibus normality test for Gaussian distribution. For comparison between GPA patients and HCs the unpaired t-test was used for data with Gaussian distribution and the Mann-Whitney U test for data without Gaussian distribution. For intra-individual comparison between samples treated with or without ShK-186, the paired t test was used for data with Gaussian distribution and the Wilcoxon singed rank test for data without Gaussian distribution. Differences were considered statistically significant at 2-sided *P*-values equal to or less than 0.05.

RESULTS

T cell subset distribution in peripheral blood of GPA patients in remission

We first assessed the distribution of $CD4^+$ T cell subsets in the peripheral blood of GPA patients in remission and HCs. $CD4^+$ T cell subsets were identified based on the surface expression of CD45RO and CCR7 and divided into $CD4^+$ T_{NAIVE} cells ($CD45RO^-CCR7^+$), $CD4^+$ T_{CM} ($CD45RO^+CCR7^+$), $CD4^+$ T_{EM} cells ($CD45RO^+CCR7^-$) and $CD4^+$ terminal differentiated cells (T_{TD}, $CD45RO^-CCR7^-$) (figure 1A). We found that the percentage of circulating $CD4^+$ T_{EM} cells from GPA patients was significantly higher compared to HCs (figure 1B). The percentage of circulating $CD4^+$ T_{NAIVE} cells was significantly lower in GPA patients compared to HCs, whereas the percentage of $CD4^+$ T_{CM} cells did not differ. In addition, the percentage of circulating $CD4^+$ T_{TD} cells was significantly higher in GPA patients compared to HCs.

To rule out the possibility that the increased proportion of $CD4^+$ T_{EM} cells was influenced by current treatment, we compared the proportions of $CD4^+$ T_{EM} cells between GPA patients off treatment and GPA patients receiving immunosuppressive maintenance therapy. No significant differences were found between the treated and untreated patient group (data not shown).

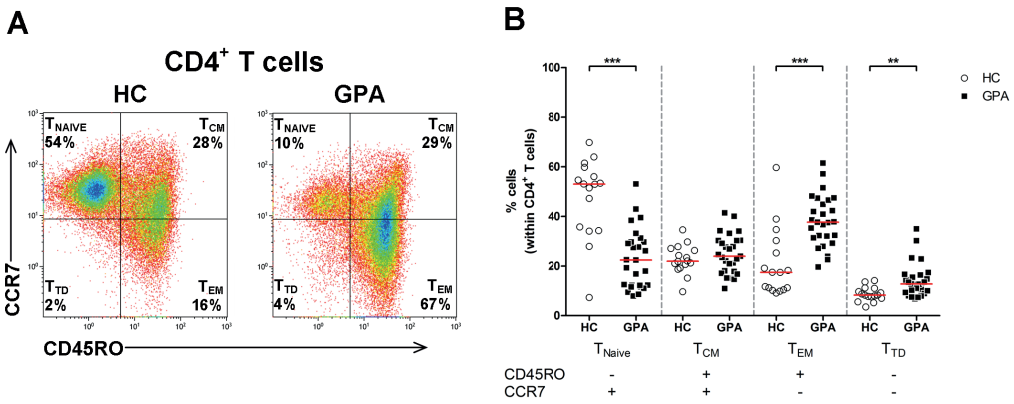

Figure 1 | Increased percentage of circulating CD4$^+$ T_{EM} cells in GPA patients.

A) Representative flow cytometry dot plots of CD45RO and CCR7 expression to identify four CD4$^+$ T cell subsets in the peripheral blood of a GPA patient in remission (right plot) and a HC (left plot). B) Percentages of CD45RO$^-$CCR7$^+$ (T_{NAIVE}), CD45RO$^+$CCR7$^+$ (T_{CM}), CD45RO$^+$CCR7$^-$ (T_{EM}) and CD45RO$^-$CCR7$^-$ (T_{TD}) subsets within the CD4$^+$ T cell population in peripheral blood of GPA patient in remission (filled squares; *n*=27) and HCs (open circles; *n*=16). Horizontal bar represent median percentage. **p<0.01, ***p<0.001 versus HCs.

Increased intracellular pro-inflammatory T cell cytokine production in GPA patients

Effector T cells produce pro-inflammatory cytokines (such as IL-4, IL-17, IL-21 TNFα, and IFNγ) that are presumed to be involved in the disease pathogenesis of GPA [10, 13]. Therefore, we next analyzed the pro-inflammatory cytokine prolife of CD4$^+$ T_H cell from GPA patients and HCs. Fresh peripheral blood samples of GPA patients in remission and HCs were stimulated *in vitro* with or without PMA and CaI for 16 hours. In all samples the production of intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ was determined in CD4$^+$ T_H cells by flow cytometry (figure 2A). As shown in figure 2, the expression of all pro-inflammatory cytokines within CD4$^+$ T_H cells was significantly higher in GPA patients compared to HCs.

Of note, it has become evident that CD4$^+$ T_H cells may produce additional cytokines besides their signature cytokine. For example, T_H1 cells may produce IL-17 in addition to their signature cytokine IFNγ, and T_H17 cells produce IL-21 in addition to their signature cytokine IL-17 [24]. We, therefore, assessed the proportion of CD4$^+$ T_H cells producing 2 cytokines (TNFα$^+$IFNγ$^+$, IFNγ$^+$IL-17$^+$, and IL-17$^+$IL-21$^+$). As shown in figure 2B, CD4$^+$ T_H cells from GPA patients in remission produce significant higher percentages of TNFα$^+$IFNγ$^+$, IFNγ$^+$IL-17$^+$, and IL-17$^+$IL-21$^+$ cytokines as compared to CD4$^+$ T_H cells from HCs. Overall these results demonstrate the pro-inflammatory nature of the CD4$^+$ T_H cells in patients with GPA.

ShK-186 normalized the production of cytokines in CD4$^+$ T_H cells from GPA patients.

Next, we questioned whether the pro-inflammatory cytokine production could be regulated by Kv1.3 channel blockade, using the highly potent Kv1.3 peptide blocker ShK-186. To this end, we stimulated peripheral blood samples of GPA patients in the presence and absence of ShK-186

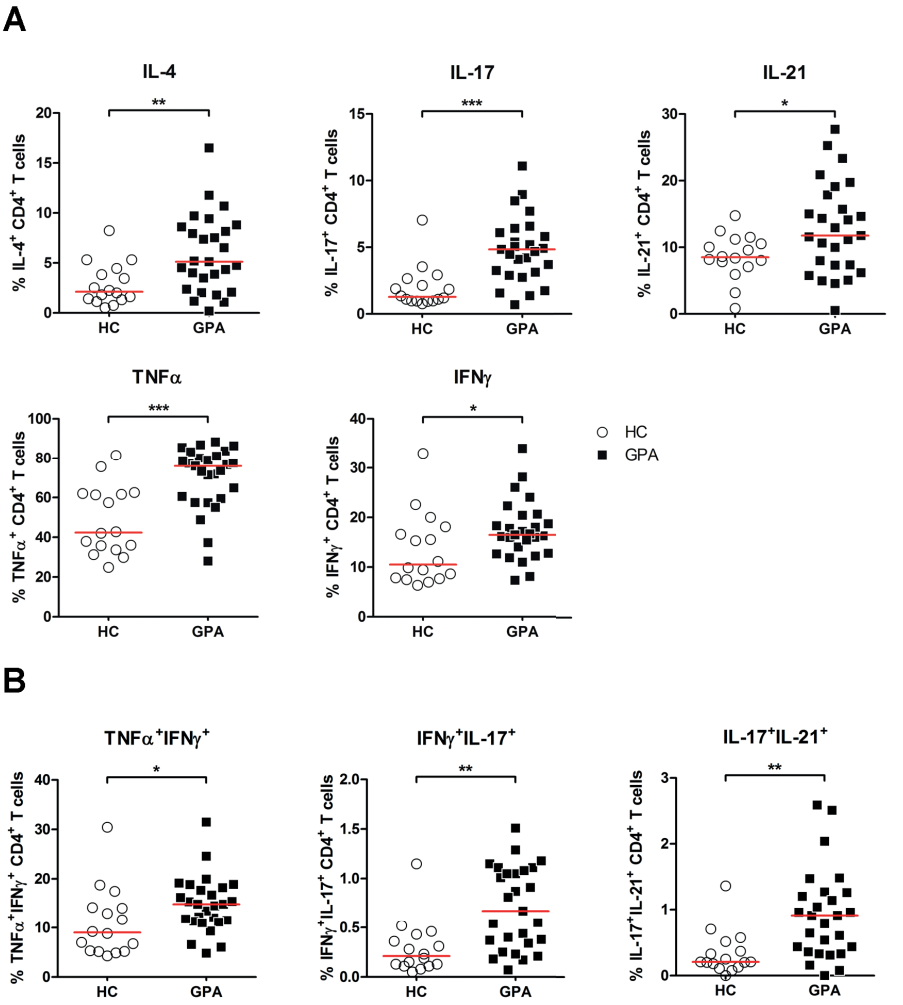

Figure 2 | Higher percentage of intracellular cytokine production in circulating CD4$^+$ T$_H$ cells from GPA patients.

Peripheral blood of GPA patients and HCs was stimulated with PMA and Cal and analyzed with flow cytometry for intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ cytokine expression. A) Percentages of IL-4$^+$, IL-17$^+$ IL-21$^+$, TNFα$^+$, and IFNγ$^+$ CD4$^+$ T$_H$ cells from GPA patient in remission (filled squares; *n*=27) and HCs (open circles; *n*=16). B) Percentages of TNFα$^+$IFNγ$^+$, IFNγ$^+$IL-17$^+$, and IL-17$^+$IL-21$^+$ within CD4$^+$ T$_H$ cells from GPA patient in remission (filled squares; *n*=27) and HCs (open circles; *n*=16). Horizontal bar represent median percentage. *p<0.05, **p<0.01, ***p<0.001 versus HCs.

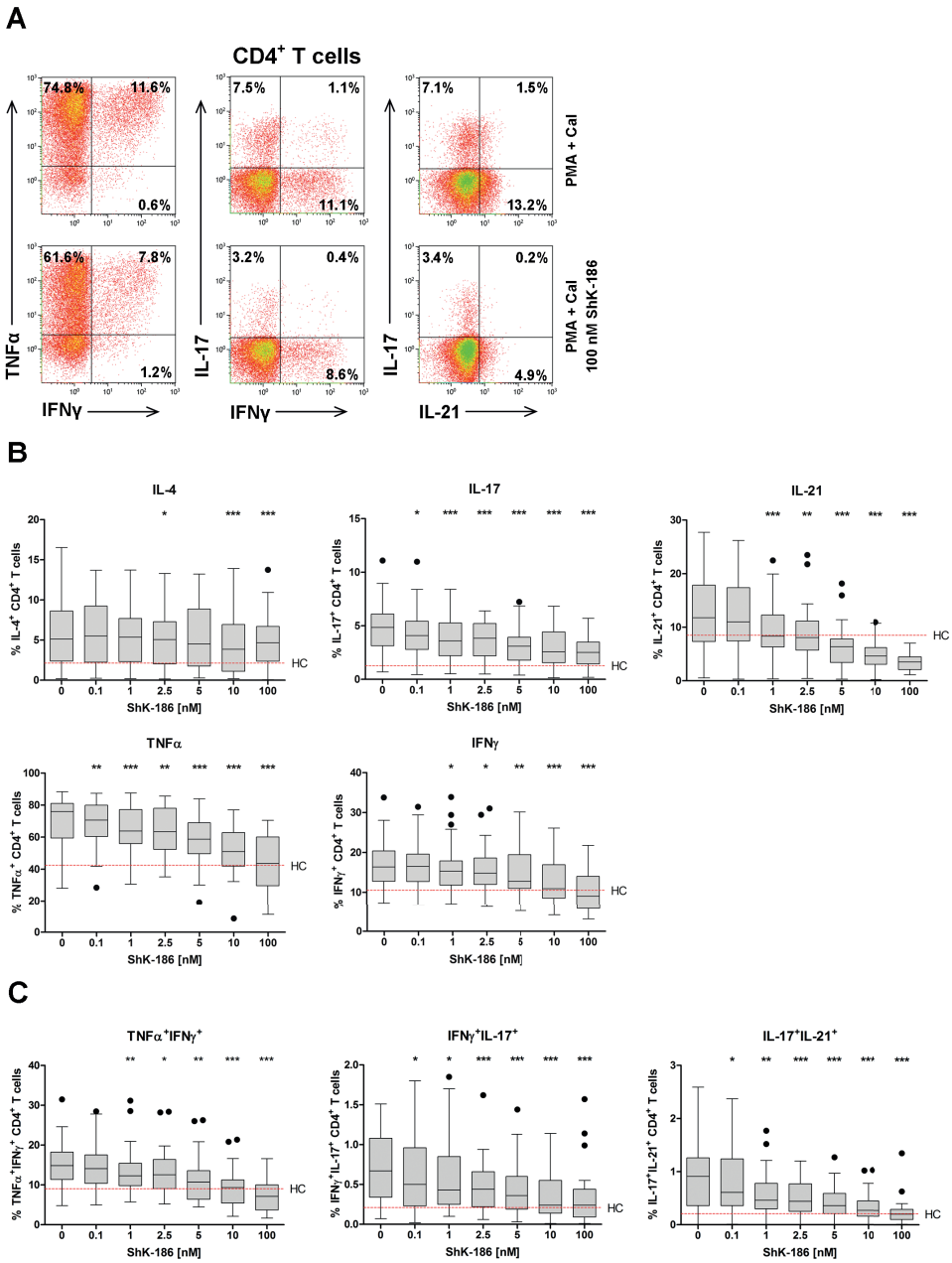

Figure 3 | Dose dependent suppression of pro-inflammatory cytokines by ShK-186 in CD4$^+$ T_H cells from GPA patients.

Peripheral blood of GPA patients and HCs was stimulated with PMA and Cal with and without increasing concentrations of ShK-186. Intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ cytokine production in CD4$^+$ T_H cells was analyzed using flow cytometry. A) Representative flow cytometry dot plots of cytokine expression within CD4$^+$ T_H cells after stimulation in the presence (lower panels) and absence (upper panels) of ShK-186 from a GPA patient in remission. B) Percentages of cytokine producing CD4$^+$ T_H cells after stimulation in the presence and absence of ShK-186 from GPA patients in remission (grey box and whiskers; *n*=27). C) Percentages of TNFα$^+$IFNγ$^+$, IFNγ$^+$IL-17$^+$, and IL-17$^+$IL-21$^+$ within CD4$^+$ T_H cells after stimulation in the presence and absence of ShK-186 from GPA patients in remission (grey box and whiskers; *n*=27). Box and whiskers plots (tukey), boxes represent median values and interquartile range. Red horizontal dashed line represent median percentage of cytokine production by CD4$^+$ T_H cells from HCs. *p<0.05, **p<0.01, ***p<0.001 versus stimulated CD4$^+$ T_H cells without ShK-186.

and analyzed the intracellular cytokine production of IL-4, IL-17, IL-21, TNFα, and IFNγ in $CD4^+$ T_H cells from GPA patients (supplementary figure 1). As shown in figure 3, addition of ShK-186 to stimulated cell cultures significantly reduced the production of IL-17, IL-21, TNFα, and IFNγ in $CD4^+$ T_H cells from GPA patients. The effect of ShK-186 on the production of IL-17, IL-21, TNFα and IFNγ was dose dependent (figure 3B). Interestingly, the production of IL-17, IL-21, TNFα and IFNγ in $CD4^+$ T_H cells was normalized to median cytokine levels detected in HC. Remarkably, the suppressive effect of ShK-186 on IL-4 production was less pronounced.

In addition, the percentage of $CD4^+$ T_H cells producing $TNFα^+IFNγ^+$, $IFNγ^+IL\text{-}17^+$, and $IL\text{-}17^+IL\text{-}21^+$ were significantly suppressed by ShK-186 in a dose dependent manner (figure 3C).

ShK-186 inhibits cytokine production of $CD4^+$ T_{EM} cells

As described previously, $CD4^+$ T_{EM} cells express significantly higher numbers of Kv1.3 channels on their plasma membrane compared to $CD4^+$ T_{NAIVE} cells and $CD4^+$ T_{CM} cells [19, 20]. Therefore $CD4^+$ T_{EM} cells are the pronounced target for ShK-186. To study if Kv1.3 channel blockade by ShK-186 selectively targets the cytokine production of $CD4^+$ T_{EM} cells, we tested the effect of ShK-186 on FACS sorted purified $CD4^+$ $T_{NAIVE,}$ $CD4^+$ T_{CM}, $CD4^+$ T_{EM}, and $CD4^+$ T_{TD} cells (figure 4A). First, we observed that the pro-inflammatory cytokine production of IL-4, IL-17, and IFNγ after *in vitro* stimulation was significantly increased in $CD4^+$ T_{EM} cells compared to the other $CD4^+$ T cell subsets ($CD4^+$ T_{NAIVE}, $CD4^+$ T_{CM}, and $CD4^+$ T_{TD} cells) (figure 4). IL-21 was significantly increased in $CD4^+$ T_{EM} cells compared to $CD4^+$ T_{NAIVE} and $CD4^+$ T_{TD} cells, whereas no difference was observed compared to $CD4^+$ T_{CM} cells. TNFα was produced by all $CD4^+$ T cell subsets, although the production of TNFα by $CD4^+$ T_{EM} cells showed the highest expression levels of TNFα compared to intermediate expression levels of TNFα by $CD4^+$ T_{NAIVE}, $CD4^+$ T_{CM}, and $CD4^+$ T_{TD} cells. Overall, *in vitro* stimulation with PMA and CaI showed that $CD4^+$ T_{EM} cells are the major producer of pro-inflammatory cytokines in comparison to other $CD4^+$ T cell subsets (figure 4B). Addition of ShK-186 inhibited $CD4^+$ T_{EM} cells from producing IL-4, IL-17, TNFα, and IFNγ in a dose depended manner, whereas such an effect was less pronounced in the $CD4^+$ T_{NAIVE}, $CD4^+$ T_{CM}, and $CD4^+$ T_{TD} cells (figure 4B). In contrast, the production of IL-21 after addition of ShK-186 in the four different $CD4^+$ T cells subsets showed a different pattern compared to the other cytokines. Interestingly, we observed that $TNFα^+IFNγ^+$ $CD4^+$ T_H cells were predominantly present within the $CD4^+$ T_{EM} subset. Addition of ShK-186 demonstrated a significant dose dependent inhibition of $TNFα^+IFNγ^+$ production by $CD4^+$ T_{EM} cells compared to the other $CD4^+$ T cell subsets (figure 4B).

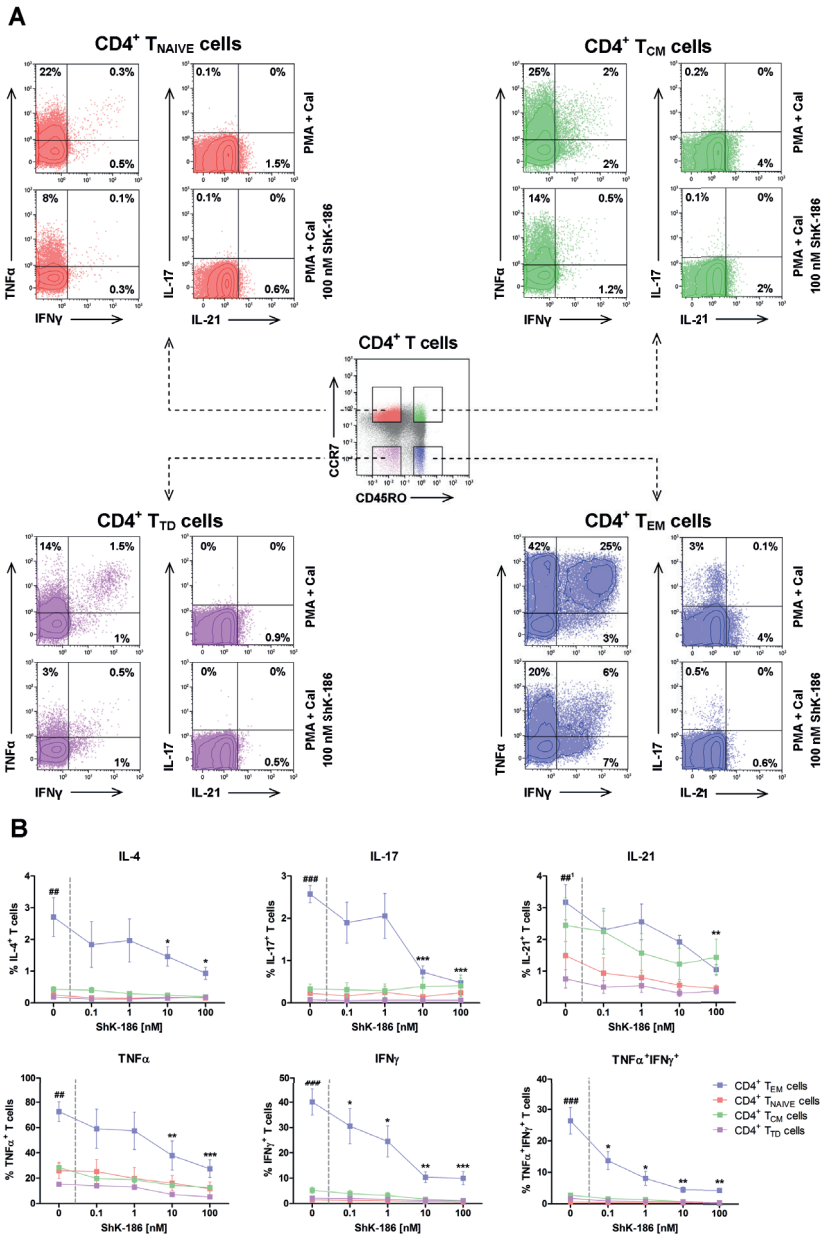

Figure 4 | ShK-186 inhibits the pro-inflammatory cytokine production of CD4$^+$ T$_{EM}$ cells.

CD4$^+$ T cells subsets (i.e. CD4$^+$ T$_{NAIVE}$, CD4$^+$ T$_{CM}$, CD4$^+$ T$_{EM}$, and CD4$^+$ T$_{TD}$ cells) were isolated from PBMCs of HCs followed by stimulation with PMA and Cal with and without increasing concentrations of ShK-186. Intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ cytokine production in the CD4$^+$ T cell subsets was analyzed using flow cytometry. A) Representative flow cytometry dot plots of CD4$^+$ T cells subsets based on surface expression of CD45RO and CCR7 (center dot plot), and the cytokine expression within CD4$^+$ T$_{NAIVE}$ cells (upper left, red), CD4$^+$ T$_{CM}$ cells (upper right, green), CD4$^+$ T$_{TD}$ cells (lower left, purple), and CD4$^+$ T$_{EM}$ cells (lower right, blue) after stimulation in the presence and absence of ShK-186 from a HC. B) Percentages of intracellular cytokine production in CD4$^+$ T$_{NAIVE}$ cells (red symbol & line), CD4$^+$ T$_{CM}$ cells (green symbol & line), CD4$^+$ T$_{TD}$ (purple symbol & line), and CD4$^+$ T$_{EM}$ cells (blue symbol & line) after stimulation in the presence and absence of ShK-186 from HCs (*n=5*). Data represent mean values ± SEM. ##p<0.01, ###p<0.001 indicate CD4$^+$ T$_{EM}$ cells versus CD4$^+$ T$_{NAIVE}$ cells, CD4$^+$ T$_{CM}$ cells, and CD4$^+$ T$_{TD}$ cells. *p<0.05, **p<0.01, ***p<0.001 indicate CD4$^+$ T$_{EM}$ cells with vs without ShK-186 [1]: IL-21 production in CD4$^+$ T$_{EM}$ cells is only significant different compared to CD4$^+$ T$_{NAIVE}$ and CD4$^+$ T$_{TD}$ cells and not significant different compared to CD4$^+$ T$_{CM}$ cells.

DISCUSSION

In the present study, we show that pro-inflammatory cytokine producing $CD4^+$ T_H cells are proportional increased in the circulation of GPA patients in remission compared to HCs. We found that *in vitro* pharmacological blockade of Kv1.3 channels using ShK-186 decreased the production of pro-inflammatory cytokines including IL-17, IL-21, TNFα, and IFNγ of $CD4^+$ T cells from GPA patients. Importantly, ShK-186 treatment did not completely inhibit cytokine production but rather normalized the production of these pro-inflammatory cytokines to the level seen in $CD4^+$ T_H cells from healthy controls. Furthermore, addition of ShK-186 predominantly affected cytokine production of $CD4^+$ T_{EM} cells without impairing cytokine production of the other $CD4^+$ T cell subsets (i.e. $CD4^+$ T_{NAIVE}, $CD4^+$ T_{CM}, and $CD4^+$ T_{TD} cells).

Our observation that $CD4^+$ T_H cells from GPA patients display an increased pro-inflammatory cytokine prolife compared to cells from HCs is consistent with previous reports demonstrating increased production of IFNγ and TNFα by PBMCs and $CD4^+$ T_H cells of GPA patients [10, 25, 26]. In addition, we and others have demonstrated that circulating IL-17 and IL-21 producing $CD4^+$ T_H cells are significantly increased in GPA patients even in remission [15-17].

Next, we demonstrated that the increase in pro-inflammatory cytokine production in $CD4^+$ T_H cells from GPA patients can be prevented by ShK-186 treatment. These data are in line with previous reports showing that ShK-186 preferentially suppresses production of IL-2, IFNγ, TNFα from synovial T cells (mainly consisting of $CD4^+$ T_{EM} cells) of RA patients [23]. In addition, Chi et al. have demonstrated that ShK-186 suppresses cytokine production in human T cells from whole blood [27]. Similar to our observations, these authors reported that Shk-186 was most effective in suppressing the production of IL-2 followed by IFNγ and IL-17 but had a minor effect only on IL-4 production. Interestingly, it has been shown that TCR induced Ca^{2+} signaling is lower in T_H2 cells than in T_H1, T_H17 or naïve T cells [28, 29] suggesting that Kv1.3 mediated T cell activation is differently regulated not only in T cell subsets (i.e. T_{NAIVE}, T_{CM} and T_{EM}) but also between different T cell phenotypes. This could explain the fact that blocking Kv1.3 channels using ShK-186 has a more pronounced effect on the pro-inflammatory cytokines IFNγ and IL-17 compared to IL-4.

Using sorted $CD4^+$ T cell subsets, we observed that cytokine production is most effectively suppressed by ShK-186 in $CD4^+$ T_{EM} cells. This can be explained by the fact that activation of T cells has differential effects on the expression of potassium channels in different T cells subsets. $CD4^+$ T_{NAIVE} and $CD4^+$ T_{CM} cells preferentially up-regulate the Ca^{2+}-activated potassium KCa3.1 channel while $CD4^+$ T_{EM} cells preferentially increase their Kv1.3 expression [20]. This switch in channel expression significantly affects responsiveness of T cell subsets to Kv1.3 and KCa3.1 blockers, $CD4^+$ T_{EM} cells being highly sensitive to Kv1.3 channel blockers and $CD4^+$ T_{NAIVE}/T_{CM} cells being more sensitive to KCa3.1 channel blockers.

In addition, ShK analogs have shown similar effects on rat T cells in various immune-mediated inflammatory disease models. In these studies, ShK analogs showed efficacy in preventing and ameliorating acute experimental autoimmune encephalomyelitis (EAE, a model of multiple sclerosis) and pristine-induced arthritis in rats [23, 30]. Moreover, in a rat model of anti-glomerular

basement membrane (GBM) glomerulonephritis, the majority of $CD4^+$ T cells infiltrating the kidney were $Kv1.3^{high}$ T_{EM} cells [31]. Rats treated with a Kv1.3 blocker developed less proteinuria and had fewer crescentic glomeruli than rats treated with placebo. ShK-186 may therefore be useful in the treatment of autoimmune kidney disease like GPA.

In this study, blood samples from GPA patients in remission were evaluated for the effect of ShK-186, and not in those with active disease. We have previously shown that during active disease $CD4^+$ T_{EM} cells appear to migrate towards inflamed tissues [13]. Analysis of ShK-186 effect on circulating $CD4^+$ T_H cells in GPA patients with active disease will exclude cells migrated to inflamed tissue which are, probably, the most relevant cells. Therefore, studying samples from patients in remission seems more relevant for this analysis.

Apart from $CD4^+$ T_{EM} cells, Kv1.3 channels are expressed in several tissues in the body including the kidney, liver and the central nervous system. Therefore, one may argue that toxic side effects are a potential concern when using Kv1.3 channel blockers. However, Kv1.3 blockers (especially the ShK analogs) have been shown to have an excellent safety prolife in animal models [22, 23, 32]. ShK-186 was reported to exhibit no perceptible *in vitro* toxicity, was negative in the amses test, and had no effect on cardiac parameters [22]. Furthermore, repeated subcutaneous administration of ShK-186 in rats did not cause clinical toxicity as evidenced by normal blood cell counts and serum chemistry parameters, and no signs of histopathological changes in various tissues [22, 23]. Moreover, *in vivo* studies have demonstrated that the efficacy of ShK186 can be achieved without general immunosuppression [32]. In rats, administration of ShK-186 did not compromise the protective immune response to acute viral (Influenza) or bacterial (*Chlamydia*) infections at pharmacological doses that did ameliorate autoimmune diseases [32]. Importantly, ShK-186 has completed phase 1a (NCT02446340) and 1b (NCT02435342) clinical studies showing the blocker is well tolerated and has a good safety profile. The phase 1b trial was completed with psoriasis patients and demonstrated that ShK-186 was successful in reducing inflammatory cytokines involved in autoimmune processes.

Therapies targeting $CD4^+$ T_{EM} cells via blocking Kv1.3 channels may have an advantage over current therapies in GPA because $CD4^+$ T_{NAIVE} and $CD4^+$ T_{CM} would escape the inhibition by ShK-186. Leaving T_{NAIVE} and T_{CM} $CD4^+$ T cells unimpaired. GPA patients treated with ShK-186 would therefore be able to preserve protective immune responses against most pathogenic challenges. On the other hand, a potential disadvantage of Kv1.3 blockade is that it likely suppresses all $CD4^+$ T_{EM} cells thereby affecting immune responses against chronic infections. However, as demonstrated here, it may be possible to titrate ShK-186 to a dose at which it normalizes, but does not completely suppress, $CD4^+$ T_{EM} cell responses. Moreover, the Kv1.3 blocker is reversible and therapy could be paused in the event of an acute infection, unlike current treatments in GPA (i.e. cyclophosphamide, high dose corticosteroids and rituximab) which take several months to subside.

Conclusion

In conclusion, the data presented here demonstrate that the Kv1.3 blocker ShK-186 suppresses pro-inflammatory cytokine production in human $CD4^+$ T_H cells from GPA patients. Furthermore, we showed that cytokine production in $CD4^+$ T_H cell by GPA patients can be normalized to cytokine levels of HCs and that ShK-186 predominantly inhibited the cytokine expression in $CD4^+$ T_{EM} cells. These findings support the potential of selective Kv1.3 blockade as a therapeutic strategy for GPA patients.

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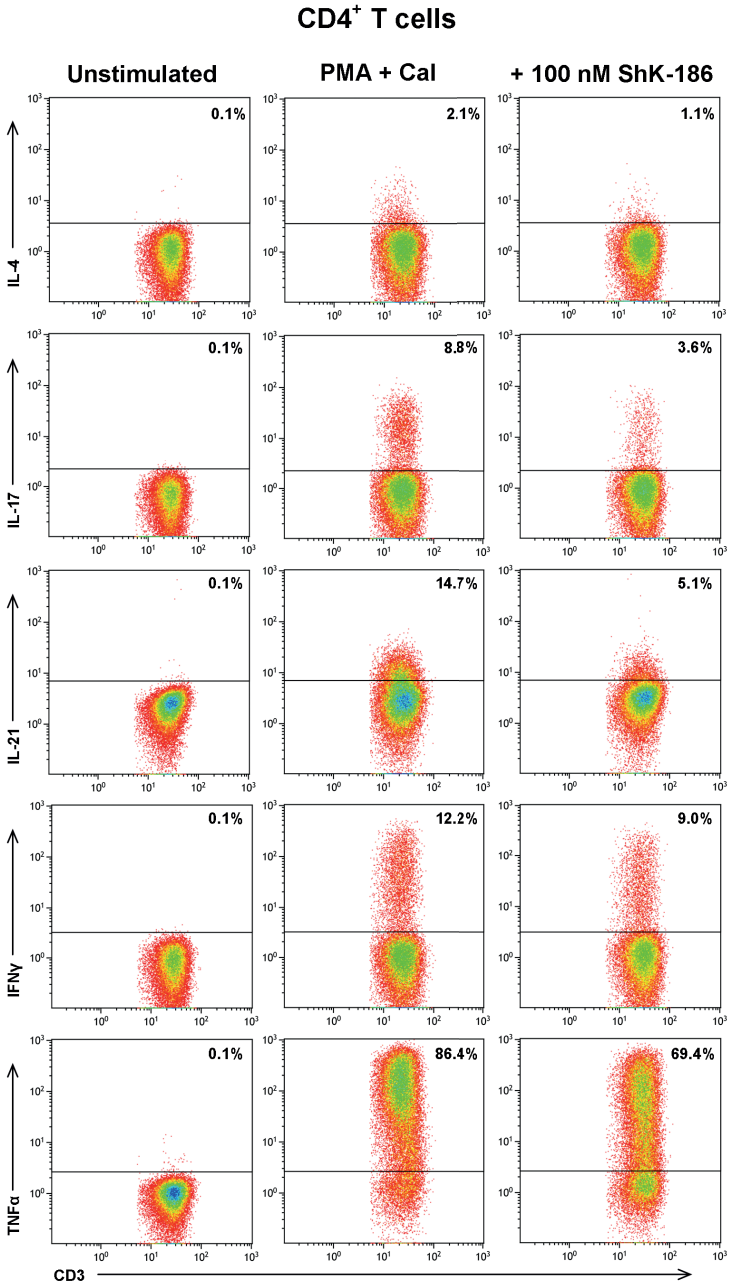
SUPPLEMENTARY MATERIAL



Supplementary figure 1 | Flow cytometry analysis of intracellular cytokine production in CD4$^+$ T$_H$ cells.

Representative flow cytometry dot plots of intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ cytokine production within CD4$^+$ T$_H$ cells without stimulation (left column) and after stimulation in the absence (middle column), and presence (right column) of ShK-186 from a GPA patient in remission.